Cold stress: **Manipulating freezing tolerance in plants**

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A single transcription factor can trigger induction of the freezing-tolerant state in *Arabidopsis*. Is such a factor all that is lacking in non-hardy plants?

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When cells find themselves in freezing conditions, they lose water to extracellular ice by osmosis, and so acquire elevated solute concentrations — a situation equivalent to desiccation. They also become surrounded by an inflexible solid capable of causing mechanical damage and obstructing gas exchange. It seems a dire predicament. But there are freezing-tolerant species, not just of unicellular extremophiles but even among 'higher' animals (arthropods and vertebrates) and plants (angiosperms and gymnosperms). In plants, such tolerance is called frost-hardiness: the agrarian term reflects the fact that its practical significance has long been recognized.

Hardy plants are not freezing-tolerant all the time. In summer, they are sensitive much like non-hardy species, but they develop tolerance when exposed to the cooler temperatures that presage the occurrence of frost. This adaptive change, occurring on a timescale of days or weeks, is known as cold acclimation. The phenomenon suggests an avenue for investigation. What occurs during cold acclimation must be responsible for freezing tolerance: find out what changes, and you should know what makes the difference.

During cold acclimation, the expression of many genes is up-regulated, and that of some down-regulated. Several groups have examined these changes to assemble a detailed, though not yet exhaustive, description of the molecular genetic changes occurring during cold acclimation [1]. Surely this must lead to a specific understanding of what makes a plant freezing-tolerant? Not quite yet. Some changes in gene expression may simply be adaptations to life at low temperatures and have nothing to do with freezing. Rice, for example, is a non-hardy species par excellence, but it shows similar changes at low temperatures.

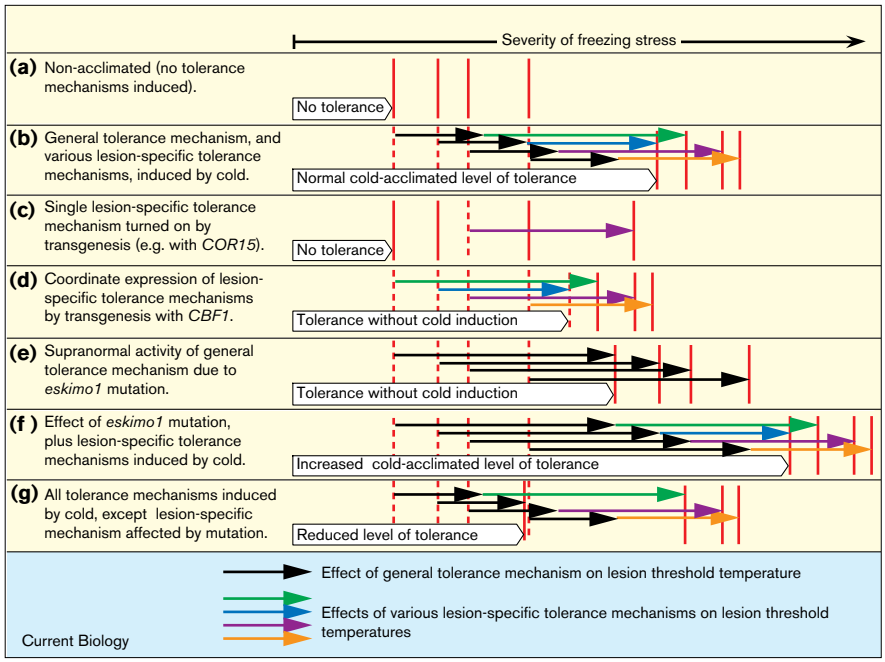
All attempts — often unreported in the literature — to boost freezing tolerance by overexpressing individual cold-inducible genes have failed. Nor have attempts to inactivate specific endogenous genes, admittedly somewhat hampered by a reliance on antisense and cosuppression strategies, so far demonstrated that any cold-inducible gene is essential for freezing tolerance. But in a dramatic recent result, Thomashow and colleagues [2] have found that the coordinate expression of a whole group of cold-induced genes induces freezing tolerance in *Arabidopsis thaliana*. This was achieved by overexpressing the transcription factor CBF1, which binds specifically to the so-called 'CRT/DRE element' that occurs in the promoters of cold-inducible genes. With this observation we have gone from not even knowing that cold-induced genes are necessary for freezing tolerance, to knowing that their expression can be sufficient.

The ability to simulate cold acclimation immediately suggests applications in crop improvement. Prospects are excellent for controlling the timing and tissue-specificity of freezing tolerance in hardy species. This would have application in crops that are subject to frost during seasons in which freezing tolerance is not naturally induced. Global warming will increase the need for this, as its effect on seasonal mean temperatures will be greater than its effect on seasonal minimums. A second application would be in enabling plant parts produced in summer to be shipped under mild freezing temperatures. The storage life and so the distribution range of fresh vegetables is highly temperature-dependent, and thus an ability to withstand mild freezing would add post-harvest value.

What are the prospects for conferring tolerance on non-hardy species by means of the transcriptional activator? It will work only if non-hardy plants possess the appropriate structural genes to prevent freezing injury, with promoters able to respond to the transcriptional activator. At first sight this seems a tall order. The structural genes may well be present, however, maintained even in non-hardy species by natural selection for desiccation-tolerance of the seed. It is possible that they could be coordinately regulated by a single transcription factor, as such a system could likewise have been maintained by selection at the seed stage. So a transcriptional activator could be the magic bullet to make tender plants hardy.

But enough of optimism. There is also evidence to suggest the opposite view. Drought and/or abscisic acid (ABA) can induce hardy species to become freezing-tolerant. Not so with most non-hardy plants, even though dehydration and ABA are the cues for them to induce dehydration-tolerance in the tissues of the seed. Or consider rye, which is more freezing-tolerant than *Arabidopsis*, surviving temperatures 15°C lower. Does rye merely have a more effective

Figure 1



A speculative model of how tolerance mechanisms affect lesion thresholds and thereby determine the maximum severity of freezing stress that can be survived. Temperature thresholds for four hypothetical cold-induced lesions (red vertical lines) are moved toward lower temperatures (rightward) by two different types of tolerance mechanism. A general tolerance mechanism (black arrows) affects the thresholds of all lesions. Lesion-specific tolerance mechanisms (coloured arrows) each move the threshold of one lesion only. Effects of the two types of tolerance mechanism are assumed to combine additively. **(a,b)** The situation in non-acclimated (a) and cold-acclimated (b) wild-type *Arabidopsis* plants. **(c)** An illustration of why a single lesion-specific mechanism may have no effect on the tolerance of the whole plant. **(d)** Contrasting the effect of a single lesion-specific mechanism with that of all lesion-specific mechanisms acting together. **(e,f)** A possible basis for the observed levels of freezing tolerance in non-acclimated (e) and cold-acclimated (f) *eskimo1* plants. **(g)** A possible explanation for why mutations in lesion-specific tolerance mechanisms can be detected at the whole-plant level.

transcription factor, or is the difference in the target genes that are turned on by the transcription factor?

So there remain practical as well as academic reasons to understand not only the control, but also the mechanism, of freezing tolerance. A hardy plant must combine survival abilities for all of the various types of damage (lesions) that freezing can cause. This notion has been championed by Steponkus [3], on the basis of a powerful series of physiological experiments with rye. Each lesion will have a temperature threshold below which the severity of that type of damage becomes lethal. On the simplest interpretation, which neglects possible synergy between lesions, the highest of these thresholds will be the temperature limit below which freezing is lethal. Figure 1 incorporates this principle into a speculative model that is consistent with a variety of results.

Changes that occur during cold acclimation must shift temperature thresholds downwards, at least for the highest one or several thresholds. One change is the increase in cellular concentrations of 'compatible' osmolytes, which may be proline, betaines and/or soluble carbohydrates [4–7], according to the plant species. Compatible osmolytes may be regarded as water substitutes, maintaining a hydrophilic environment for all types of biological molecule and so generally reducing the sensitivity of molecular structures to the lack of water. At any rate, their presence certainly reduces the concentrations reached by 'incompatible' (toxic) solutes when water is removed from the cell. Compatible osmolytes are thus likely to embody a 'general' tolerance mechanism, lowering the temperature thresholds of multiple types of lesion. The *Arabidopsis eskimo1* mutant, selected for freezing tolerance in the absence of cold acclimation [8], may be tolerant by this type of general mechanism alone. The proline and sugar levels in this mutant are elevated even more than is normal for the cold-acclimated state, but it shows no alteration in the expression of CBF1-controlled genes.

There is evidence that some mechanisms of freezing tolerance are, by contrast, lesion-specific. The loss of plasma membrane material during freezing is a lesion that, in rye, is pre-empted during cold acclimation by a change in the membrane's lipid composition. Experimental manipulation has revealed the molecular species of lipid that are responsible [9]. The protective ability of these lipids is presumably limited to protection against membrane damage, and probably specific to this one lesion.

We do not know which types of injury are pre-empted by expression of most of the cold-induced genes. In fact, as noted above, for most of these genes we have no evidence of their individual contributions to freezing tolerance. There are, however, now some intriguing exceptions. Hincha and colleagues [10] have identified a cold-induced protein, dubbed cryoprotectin, that protects thylakoids against freezing damage *in vitro*. This protein affects one

type of membrane behaviour, and so makes a specific rather than general contribution to freezing tolerance. Similarly, the chloroplast-localised protein COR15a has been shown to protect whole chloroplasts against cold damage *in vivo* and *in vitro* [11]. Both its restriction to the chloroplast and the absence of any effect of its overexpression on whole-plant freezing tolerance suggest that COR15a provides protection only against a specific lesion.

Although lesion-specific mechanisms probably act independently of each other, each may also make additive contributions to the general tolerance provided by compatible osmolytes. Thus, the *eskimo1* mutant, when its cold-inducible genes were turned on naturally by cold acclimation, gained tolerance of still lower temperatures, thereby exceeding the tolerance level of the cold-acclimated wild-type plant. This result demonstrates the possibility of increasing the maximum level of freezing tolerance in a hardy plant.

Genetic approaches are at hand to discover more of the freezing tolerance mechanisms of *Arabidopsis*. Methods for obtaining mutants in cloned genes are becoming more powerful and accessible [12], so that the contributions of various cold-induced genes will soon come under scrutiny in this way. Conversely, mutants can be isolated on the basis of deficiency in freezing tolerance [13], which provides an approach capable of discovering components of freezing tolerance independently of their cold-inducibility. Also, an effective screen has been described for mutations that affect cold-inducible gene expression [14]. And lastly, Xin and Browse [8] have several more *eskimo* mutants up their sleeves which are freezing tolerant without showing elevated levels of either cold-induced proteins or proline; their elucidation is going to be interesting.

References

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